Functional characterization of *Ammonium transporter 1* (*AMT1*) in *Azolla pinnata* under acid soil stress conditions

Ph.D. Thesis Synopsis

(5000 words)

PRESIDENCY UNIVERSITY
KOLKATA

KARISHMA AGARWAL

Ph.D. Registration No.: **R-18RS18210144**

Registered on 28th June, 2019

Ph.D. Enrollment No.: **PU/PHD/DLS/2018/009**

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Summary of Work

(5000 words synopsis)

Thesis Title: Functional characterization of *Ammonium transporter 1* (*AMT1*) in *Azolla pinnata* under acid soil stress conditions

Abstract: In acidic soil, aluminium (Al^{3+}) and protons (H^{+}) are abundant and Al^{3+} ions inhibit the synthesis of several biological molecules. Very importantly, the genes responsible for nitrogen uptake are negatively regulated in plants under acid soil stress conditions. Hence, development of superior genotypes for improved nitrogen uptake under acid soil stress conditions is essential. Interestingly, Ammonium is the preferred nitrogen source by plants as it requires less energy than nitrate. Also, it is the major available form of nitrogen fixed by the cyanobacterium *Anabaena azollae* in the leaf cavity of *Azolla pinnata.* Further, Ammonium transporters (AMTs) of *Azolla* plants are regulating ammonium transport from the leaf cavity. AMTs belonging to the AMT/MEP/Rh (Ammonium Transporter/ Methylamine Permease/ Rhesus) protein superfamily, are present in the root/hair cell plasma membrane. Based on the above principles and mechanisms, we aimed to clone and overexpress the *AMT1* gene from *Azolla pinnata* in rice plants. After cloning and sequencing the *ApAMT1* gene from *Azolla*, the transgenic rice plants containing overexpressed *ApAMT1* gene were developed for its functional characterization. *In silico* analysis of ApAMT1 was performed with the help of several bioinformatics tools to evaluate its structural and positional details. Overexpression of *ApAMT1* was assessed in both wildtype and transgenic rice plants under different concentrations of ammonium nitrate to understand their regulation during nitrogen uptake. The rice transgenics with *ApAMT1* displayed significantly increased shoot or root biomass under low (0.4 mM) and normal (4.0 mM) amount of Ammonium nitrate. Under higher concentrations (20 mM) of ammonium nitrate, the shoot biomass strikingly decreased and the root biomass surprisingly increased two-fold. In continuation, the *ApAMT1* transgenics were found to increase nitrogen use efficiency (NUE) even at 200 µM concentration of Al^{3+}(pH 4.75). Our results highlight that transgenic rice plants with *ApAMT1* gene will be very important to improve the nitrogen use efficiency under acid soil stress conditions.

1.0 INTRODUCTION

Soil acidification has increased tremendously in the last decade leading to change in mineral availability to plants and increase in metal toxicity (Yadav et al., 2020; Huang et al., 2024). The toxic aluminium (Al^{3+}) ions, dominantly available at pH<5, is absorbed by the plants hampering its root growth (Matsumoto et al. 1977). In the rhizosphere, Al^{3+} ions are neutralized with organic acids released from the plants (Kochian et al., 2005; Yamaji et al., 2009; Azura et al., 2011). Nitrogen uptake and metabolism is one of the key biological processes with immense importance for plant growth and development. It is mostly regulated by various soil stress factors. The ammonium transporters (AMTs) present in the root/hair cell plasma membrane are transporters that assist in nitrogen transport in the form of ammonium (NH^{4+}), and belong to the AMT/MEP/Rh (Ammonium Transporter/Methylamine Permease/Rhesus) protein super family (Hachiya et al., 2011). Assimilation of ammonium requires less energy than nitrate, but low pH downregulates the expression of AMTs thus affecting the NUE of the plants.

Rice (*Oryza sativa*) is a staple food and one of the most important crops grown worldwide. It is grown in water-logged conditions, which causes loss of soil nutrients further reducing the pH of the soil. The ammonium transporters, responsible for nitrogen uptake, can reduce the need of excess fertilizers and also enable cultivation in acidic soil. *Azolla pinnata* is an aquatic fern that harbours nitrogen fixing cyanobacteria, *Anabaena azollae* in symbiosis (Lumpkin et al., 1980). It is either incorporated in the soil prior to paddy plantation or grown along with it to supplement the nitrogen requirement of rice thereby enhancing its growth (Graff et al., 2011). The cyanobacterial symbiont in *Azolla* can fix a significant amount of nitrogen [0.15–0.17 mg $Nh^{-1}g^{-1}$ of dry biomass]. *Azolla* plants can survive at pH ranging from 3.5 to 10 and can also accumulate different metal ions. The effects of toxic Al and low pH of the soil on regulation of AMT1 of *Azolla pinnata* for its functional characterization has not been evaluated. Hence, in this study, we planned to characterize AMT1 from *Azolla pinnata* (*ApAMT1*) by overexpressing it in *Oryza sativa* plants using transgenic methods and decipher its role under acid soil stress. However over-irrigation, improper drainage, and overexploitation of ground water have been associated with higher salt concentrations in the soil. This also hampers the uptake and utilization of nutrients by crop plants. Therefore, we also studied the relationship between salinity and nitrogen uptake by AMTs in wild type versus transgenic rice plants.

2. OBJECTIVES OF THE STUDY

i. Characterization of acid soil stress responses of *Azolla* plants in rice field under different pH conditions.

ii. Cloning and characterization of *ApAMT1* from *Azolla* plants.

iii. Overexpression of *ApAMT1* in rice plants for functional characterization.

3.0 MATERIALS AND METHODS

3.1 Plant materials

Azolla pinnata plants were sampled from Acharya Jagadish Chandra Bose Indian Botanical Garden, Howrah, West Bengal, India, and maintained in MGRL (Molecular Genetics Research Laboratory) nutrient solution after removing algae and other contaminants. IR-64 rice seeds (*Oryza sativa*) were bought from Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu.

3.2 Standardization of growth of *Azolla* and rice plants via Hydroponics experiments under low pH stress. (Hydroponics culture and Stress treatments)

Azolla pinnata plants were sub-cultured every 2 days to maintain their optimum growth rate. The rice seeds (IR64) were grown aseptically on a cotton bed under dark conditions for 2-3 days. The uniformly germinated seeds were placed on a polystyrene float in culture solution for Al and low pH stress studies. For both monoculture and intercropping experiments, 10 rice seedlings and 50 *Azolla* each were subjected to stress treatment. The hydroponic culture solution included 2%(w/v) MGRL nutrients and 200mM $CaCl_2$, except inorganic phosphorus (Kobayashi et al., 2007). Different concentrations of Al^{3+} (10 to 50 µM) were prepared from 500 mM $AlCl_3$ and added to this solution. The pH of the medium was maintained at 5.5, 5.0 and 4.75 using 1 N HCl. The solution was renewed every alternate day. The three culture groups (*Azolla pinnata*, *Oryza sativa* and mixed culture of both species) were incubated at 28±1 °C, with a light intensity of 50±5 µmol photons $m^{-2}s^{-1}$ for 16 h/day and maintained for 15 days.

3.3 Morphological Characterization of *Azolla* and Rice plants

Fresh and dry weights of treated rice and *Azolla* plants from each culture were measured. Morphological parameters including length of root and shoot, internode length, first and second leaf length were noted using a ruler and ImageJ tools.

3.3.1 Microscopic evaluation of stained tissues of *Azolla* plants

The roots of *Azolla* and rice were treated with Evans blue stain (Matsumoto and Motoda, 2012) to evaluate the dead tissues under the microscope. The root hair length was measured using ImageJ.

3.4 Biochemical assays to study the acid soil stress impact in both *Azolla* and rice plants.

Malondialdehyde content was measured from the monocropped as well as intercropped control cultures and Al treated *Azolla* and rice seedlings using 5.0 % Trichloroacetic acid (TCA) and 2-Thiobarbituric acid (TBA; 0.67%) (Duan et al., 2005). Assessment of peroxidase enzyme activity was also done from the same cultures (Parida et al., 2004) utilizing extraction buffer (50 mM potassium phosphate buffer [1.5 mL, pH 7], 1 mM EDTA [0.03 mL] and 2% [w/v] polyvinyl polypyrrolidone [PVPP] [1.47 mL]). Total chlorophyll was estimated from these cultures using methanol (Ritchie, 2008). Total malate and citrate released from the rice seedlings of both cultures for chelating Al was quantified from the hydroponic solution after 48 h of Al stress treatment (Hampp et al., 1984). Amount of Al uptake by both the plant species was also evaluated using Atomic Absorption Spectrometry (AAS) technique (Kundu et al., 2019).

3.4.1 Expression profile Analysis of *AMT1* and *STOP1* genes

The expression profile of ammonium transporter (*AMT1*) and *STOP1* genes of both the plants were studied from all experimental set-ups. Total RNA was isolated using RNA isolation kit, Nucleospin, Takara. After cDNA synthesis (PrimeScript^{TM} 1st strand cDNA synthesis kit, Takara), the reverse transcribed cDNA was used as a template for RT-PCR (Biorad).

3.5 Isolation and Cloning of Ammonium transporter gene of *Azolla pinnata*

3.5.1 Generation of Degenerate Primers

Based on the sequences of plant *AMT1* genes accessioned in "GenBank," multiple pairs of forward and reverse degenerate primers were designed from the conserved region of the gene to amplify the homologs of *Azolla pinnata.* The sequence data used in this study were collected using the keyword "AMT1". *AMT1*-specific primers were designed for 3'-RACE and 5'-RACE by following the sequence of degenerate PCR products (Sawaki et al., 2014).

3.5.2 Isolation of cDNA

RNA extraction kit from Nucleospin, Takara was used to isolate RNA from *Azolla* plants. Using oligo (dT) primers, cDNA was synthesised (PrimeScript^{TM} 1st strand cDNA synthesis kit, Takara). The full length ApAMT1 cDNA was then obtained through degenerate PCR and RACE techniques. The amplified cDNA fragments were cloned and sequenced using vector pBI121 (Takara). The isolated cDNA of *ApAMT1* was submitted to GenBank (Accession number: OP353624).

3.6 *In silico* characterization of Ammonium transporter gene of *Azolla pinnata*

A query search for orthologous genes of *AMT1* was performed in "GenBank" using the known *AMT1* gene sequences from different plant species available after performing *ApAMT1* sequence homology search on BLAST online database. Phylogenetic tree analysis and multiple sequence alignment was performed using CLC Sequence Viewer 8. Amt1 protein topology, secondary structure and Ramachandran plot analysis was predicted using various online tools. Subcellular localization of *AMT1* was estimated by WoLF PSORT. Its interaction with other proteins was studied using STRING online tool.

3.7 Standardization of Tissue Culture Plants of IR-64 Rice Seeds

IR-64 rice seeds were grown through tissue culture methods by following the protocol given by Sahoo and Tuteja, 2012 with some modifications in the shoot and root induction media. These wild type lab-cultured rice plants developed seeds in about 3-4 months in natural conditions. The composition of hormones in the various MS media is given in the table.

Callus Induction Media: 2,4-D: 2.5 mg/L   BAP: 0.15 mg/L
Shoot Induction Media: NAA: 0.5mg/L   BAP: 0.5 mg/L   Kinetin: 0.5 mg/L GA_3: 2.0 mg/L
Root Induction Media: IBA: 0.5mg/L

3.8 Agrobacterium Mediated Transformation

3.8.1 Overexpression Vector Construction

The *ApAMT1* overexpression vector was constructed by replacing the *GUS* gene of plasmid vector pBI121 (Toyobo) with *ApAMT1* gene sequence using restriction enzymes and ligase. CaMV35S promoter controlled the expression of *ApAMT1* while NOS promoter determined the expression of *NPTII* gene. The orientation of the gene was then confirmed by sequence analysis.

3.8.2 IR-64 Rice transformation

The LBA4404 strain of *Agrobacterium tumefaciens* were transformed with the *ApAMT1* overexpression vector plasmid. The surface sterilized dehusked rice seeds (70% ethanol for one minute and 0.1% $HgCl_2$ for 10 minutes) were inoculated on CIM for callus induction. The calli were then transformed with *Agrobacterium sp.* Culture and selected carefully for shoot and root induction. The regenerated transgenic plants were then hardened and harvested for seeds.

3.8.3 Molecular Analysis of Transgenic Plants and Seed Collection

PCR positive transgenic plants were chosen for molecular analysis. PureLink PlantTotal DNA Purification kit (Invitrogen) was used to isolate genomic DNA from leaves of T0, T1 (transformed) and wild type (WT) IR64 plants. For seed collection, T0 plants were maintained in plant growth chamber under 16 / 8 hrs light and dark conditions. During the

maintenance of T0 plants, the plants were irrigated with MGRL nutrient solution and no fertilizers or pesticides were supplied to the plants.

3.8.4 Expression Analysis

Real-Time Quantitative RT-PCR analysis was performed with Power SYBR Green (Applied Biosystems) and an ABI PRISM 7000 instrument (Applied Biosystems) using a diluted cDNA series to prepare standard curves of target genes and internal control.

3.9 Comparative Analysis between Control and Transgenic Rice plants

Both the wild-type (W) and transgenic (T1) IR64 seeds were surface-sterilized and placed on cotton bed under dark conditions for 2-3 days separately. The uniformly germinated seeds were placed on a polystyrene float in culture solution for Ammonium nitrate, Al and low pH stress studies. The hydroponic culture solution included 2%(w/v) MGRL nutrients and 200mM $CaCl_2$, except inorganic phosphorus (Kobayashi et al., 2007). Different concentrations of NH_4NO_3 (0.1, 1 and 10 mM) were prepared from 200mM stock solution and added to the hydroponics. The pH of the medium was maintained at 5.75 using 1 N HCl. The solution was renewed every alternate day. The two culture groups (W and T1) were incubated at 28±1 °C, with a light intensity of 50±5 µmol photons $m^{-2}s^{-1}$ for 16 h/day and maintained for 15 days. The experiment was repeated in soil for 53 days. The concentration of NH_4NO_3 was adjusted in the soil to 0.4, 4, and 20 mM at a pH of 5.75 to achieve field-like soil solution. The W and T1 seeds were also exposed to 200 µM Al^{3+} (aluminium stress) (pH 4.75) and 250 mM NaCl (salinity stress) (pH 5.75) and observed for 7 and 20 days respectively.

3.9.1 Under Different Stress Treatments

3.9.1.1 Morphological Characterization of Control and Transgenic Plants

Both the plant groups were uprooted and their root length, shoot length, secondary root length, number and length of lateral roots, fresh root and shoot weight, number and length of root hairs were measured using ruler and Dewinter Biological Binocular Microscope to evaluate variations. Finally, ImageJ software version 1.53e was used to study the minor details.

3.9.1.2 Biochemical assays to study the acid soil stress impact in Control and Transgenic Rice plants.

The WT and T1 plants were assessed for their proline concentration, total chlorophyll, and H_2O_2 level. The soil solution was filtered and their ammonium and nitrate content were estimated.

3.9.1.3 Variations in Chlorophyll Fluorescent readings

Chlorophyll-a fluorescence measurements were noted after every 7 days in dark-adapted plants using pocket PEA instrument (Plant Efficiency Analyser, Hansatech Instruments Ltd., King's Lynn, Norfolk, UK) in dark conditions. An actinic light of 3500 $\mu molm^{-2}$ s^{-1} was used with peak wavelength of 627 nm for determining fluorescence induction.

3.10 Expression profile analysis of AMT1 transgenics under different nitrogen stress conditions

Real-Time Quantitative RT-PCR analysis of WT and T1 plants under nitrogen stress was performed with Power SYBR Green (Applied Biosystems) and an ABI PRISM 7000 instrument (Applied Biosystems) using a diluted cDNA series to prepare standard curve of AMT1 gene and internal control.

3.11 Statistical Analyses

Mean values with standard errors were interpreted in all the figures. The values were calculated by parametric moods median test. Duncan's multiple range test (DMRT) was used to determine the significance between the two treatments (P=0.05, SPSS statistical programme version 12.0).

4.0 RESULTS

Objective 1

4.1 Characterization of *Azolla* and rice plants via Hydroponics experiments under different pH stress

4.1.1 Phenotypic and microscopic variations

After 15 days, Al stress critically reduced the biomass of rice in monoculture while their height and leaf length increase with *Azolla* even at 50 μM Al (**Fig. 1**). Biomass of *Azolla pinnata* in homogenous culture reduced significantly by 85% (50 μM Al) but their growth rate quadrupled at 50 μM in mixed culture (**Fig. 2a**). On exposure to Al, both plants exhibited remarkable depletion in root length, number and weight. The roots of both rice seedlings and *Azolla* became fragile with no root hair formation at higher Al. However longer roots were observed in intercropped culture (**Fig. 1c; 2e**). In comparison with monoculture, mixed cultures are favourable to both rice and *Azolla* plants with no stress phenotypes and noteworthy growth enhancement.

4.1.2 Biochemical characters of Azolla plants and rice seedlings under Al stress

4.1.2.1 Variations in MDA content and peroxidase activity

Due to Al stress, excessive MDA was produced in monocultured plants [nearly 41% in rice and 2-fold increase up to 20 μM in *Azolla* plants] proving that Al^{3+} induces oxidative stress in plants (**Fig. 3a)**. MDA production showed a remarkable decline (up to 42%) in *Azolla* of intercropped culture in comparison with monoculture **(Fig. 2b)**.

Significantly high peroxidase activity (2-fold) was recorded in rice seedlings compared to control at 40 μM Al **(Fig. 3b)**. Incorporation of *Azolla* plants decreased it by approximately 4%. Oxidative stress of *Azolla* plants also reduced when cultured with rice plants **(Fig. 2c)**.

4.1.2.2 Variations in total chlorophyll

The total chlorophyll reduced significantly by 50% in both species (in rice plants at 50 µM Al stress and in *Azolla* plants at 30 µM) whereas in rice-*Azolla* co-culture, only 30% reduction was noted in rice and about 13% in *Azolla*.

4.1.2.3 Variations in root exudates

Malate accumulated at 30 µM Al in rice monoculture, suggesting that IR-64 rice can tolerate upto 30 µM Al in acidic soils (**Fig. 4**). *Azolla's* presence decreased malate release from rice plants by 3-fold at both 30 µM and 50 µM Al. Citrate secretion occurred only during Al stress and it accumulated more in intercropped culture. Ma et al. reported Al-induced citrate secretion from roots of rice with zero citrate in absence of Al.

4.1.2.4 Aluminium accumulation

Azolla plants accumulated more Al than rice plants in both cultures. In intercropped cultures, at 10 and 30 µM, Al accumulation in rice plants is 40-50 % lower (**Table 1**).

Table 1. Variations in Aluminium content of individual and mixed cultures of rice seedlings and *Azolla pinnata.*

Aluminium content (µg/mg)	**Aluminium concentration**				
	10 µM	20µM	30µM	40µM	50µM
MONO-CULTURES					
RICE	**0.2 ± 0.023**	**0.23 ± 0.03**	**0.4± 0.021**	**0.21± 0.02**	**0.12± 0.013**
Azolla	**0.27± 0.011**	**0.32± 0.012**	**0.38± 0.013**	**0.27± 0.017**	**nd**
INTERCROPPED CULTURE					
RICE	**0.12± 0.011**	**0.14± 0.032**	**0.21± 0.01**	**0.19± 0.021**	**0.20± 0.017**
Azolla	**0.16± 0.023**	**0.18± 0.021**	**0.24± 0.031**	**0.23± 0.02**	**0.23± 0.015**

Mean values are calculated by using two different sets of plants (n=4) (**nd – not determined).**

4.1.3 Expression analysis of AMT1 and STOP1 genes

To neutralize Al toxicity, enhanced expression (2-fold) of *ApSTOP1* (in *Azolla*) (**Fig.5c)** and *OsART1* (in rice) (**Fig.5a)** genes was observed in homogeneous culture. In intercropped culture, Al treatment doubled *OsART1* gene expression with invariable

expression of *ApSTOP1* gene in comparison with control plants, thereby emphasizing the importance of *OsART1* in Al sensing.

In monoculture, AMT1 expression was limited throughout Al-treated cultures in both rice and *Azolla* plants (**Fig.5b,d**), while introducing *Azolla* plants intensified *OsAMT1* expression by ~ 2.2 folds along with an increase in *ApAMT1* expression. In mixed culture, *AMT1* expression in both species elevated even at low Al stress (10 µM) than in control, thus stating that *Azolla* plants significantly reduce the impact of Al stress on AMT1 transporters (**Fig. 6**).

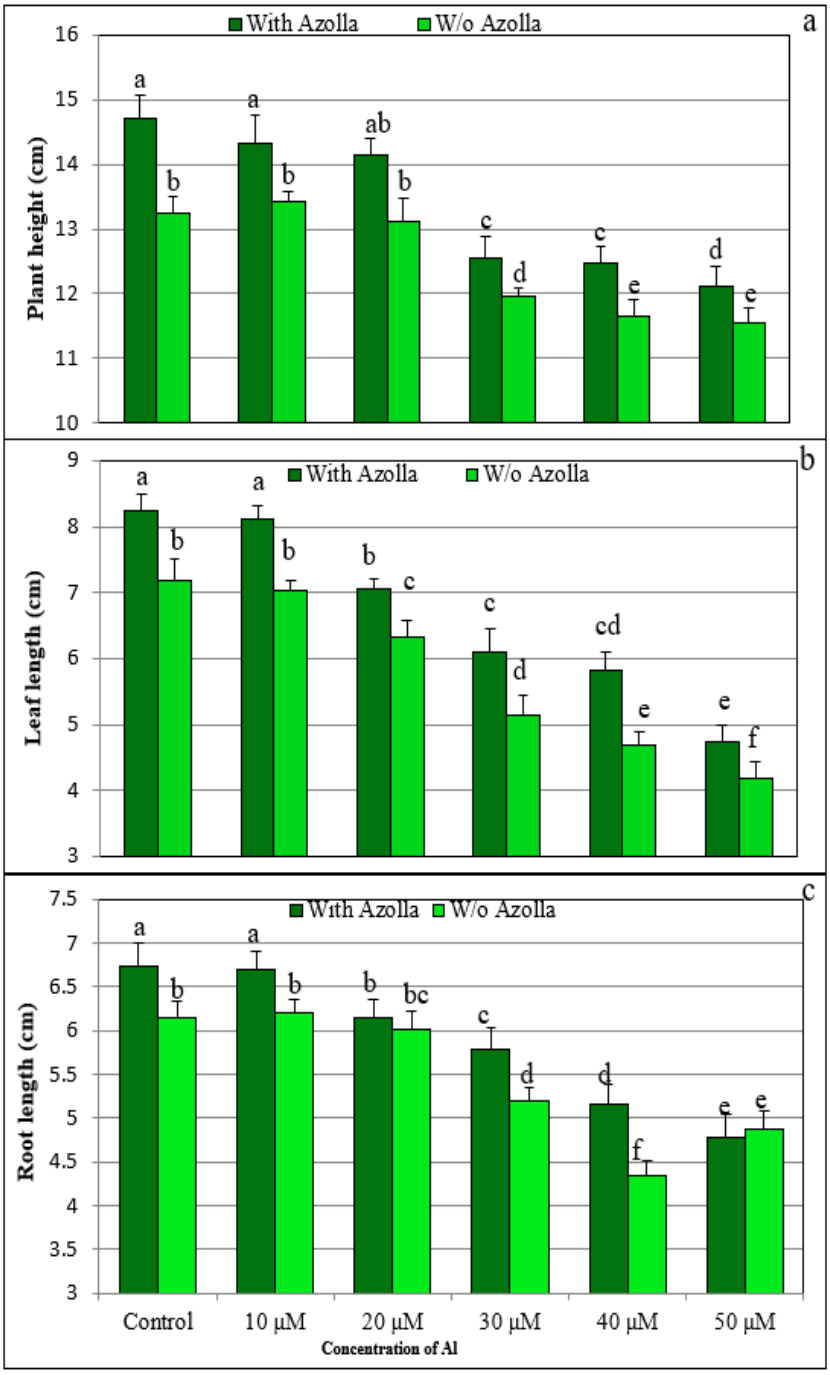


Fig. 1. Aluminium stress effects on the *total plant height (a) leaf length (b) and root length (c) of 15 days old rice seedlings* grown with or without *Azolla* plants under different Al stress (pH 4.75) condition. Each value indicates the mean ± SE (n = 12). The lower-case alphabets indicate the significance at P = 0.05 according to DMRT between mono-cultured and intercropped plants.

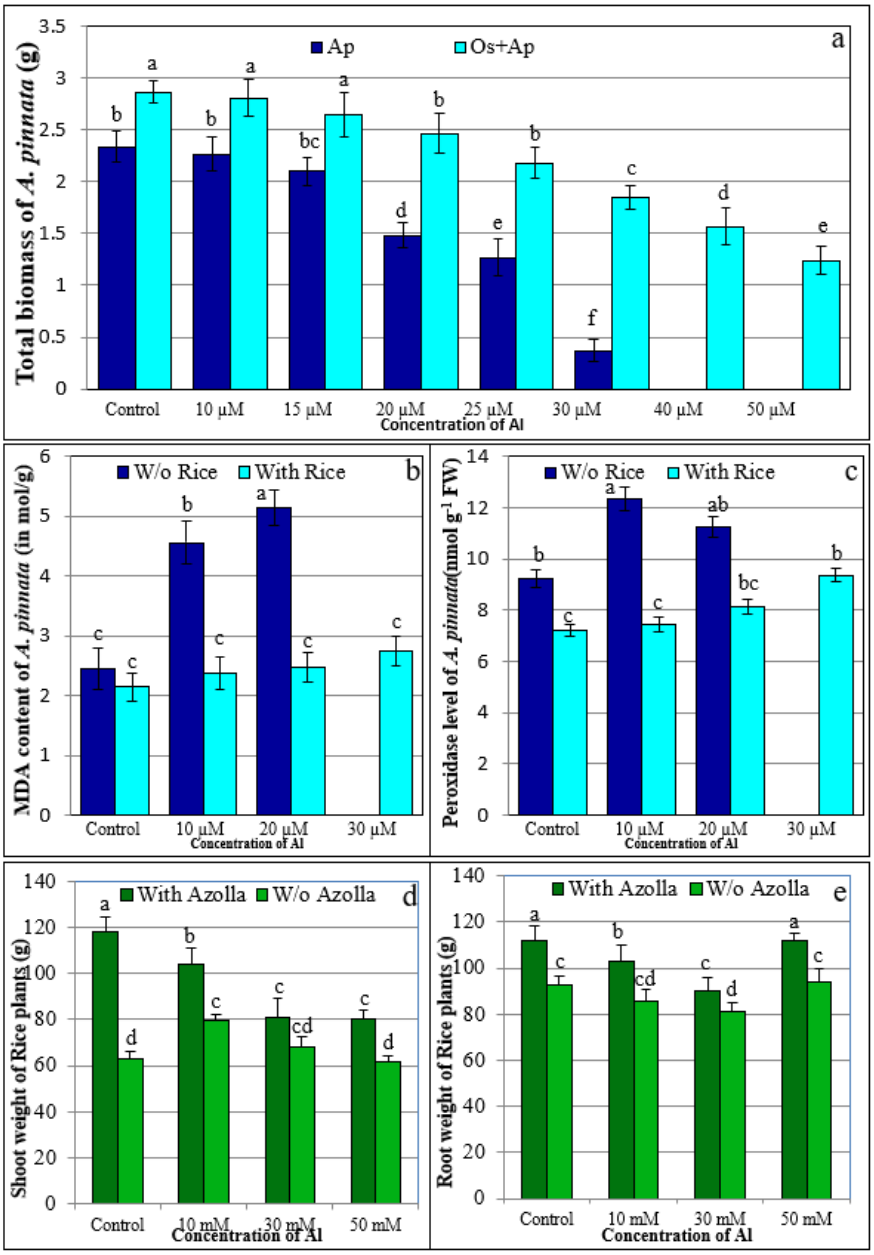


Fig. 2. Variations in the *total biomass (a), MDA content (b) and peroxidase level (c)* of 14 days old *Azolla* plants grown with or without rice plants under different Al stress and control condition. Each value indicates the mean ± SE (n = 15). Variations in *shoot weight (d) and root weight (e) of 15 days old Rice seedlings* grown with or without *Azolla* plants under different Al stress and control condition. Each value indicates the mean ± SE (n = 12). The lower-case alphabets indicate the significance at P = 0.05 according to DMRT between mono-cultured and intercropped plants.

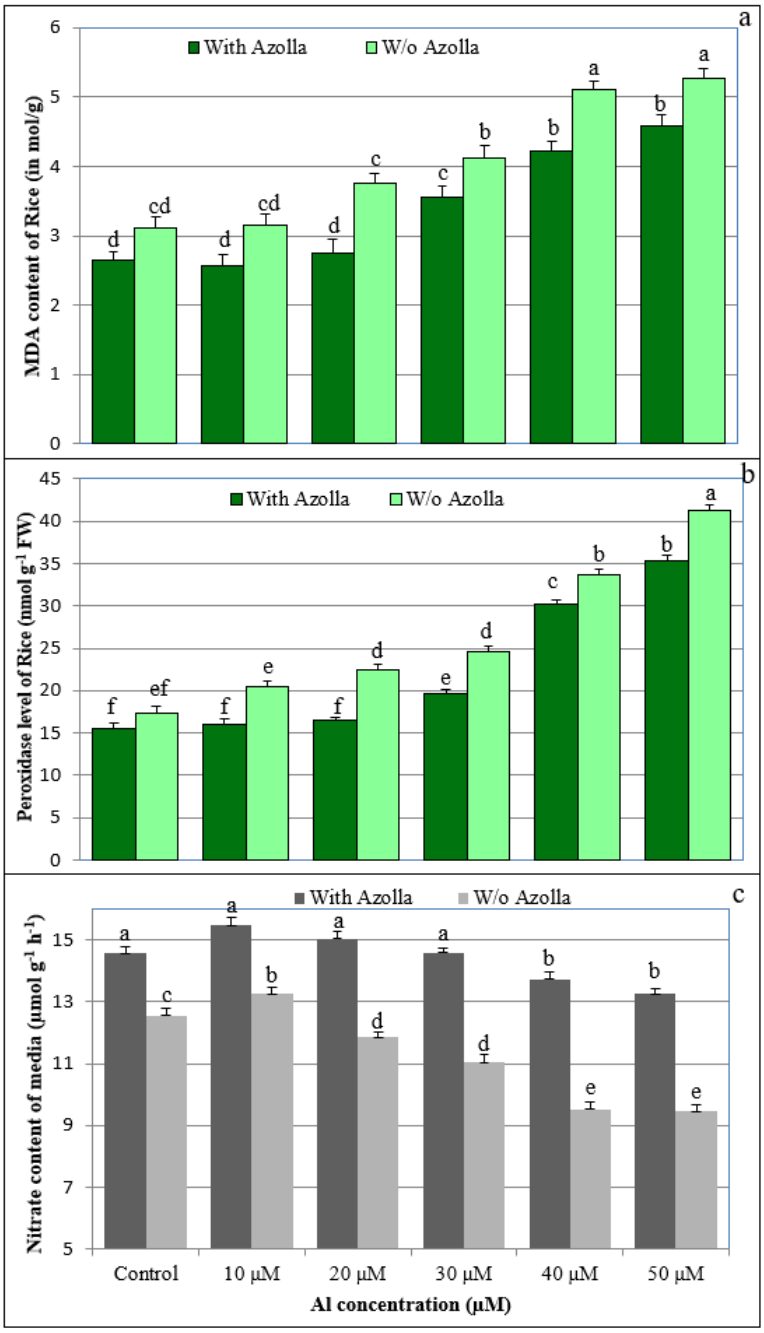


Fig. 3. Variations in the total MDA content (a) and peroxidase level (b) of rice seedlings grown with or without *Azolla* plants under different Al stress condition after 15 days of treatment. Further, total nitrate content (c) was measured from the hydroponics media. Each value indicates the mean ± SE (n = 5). The lower-case alphabets indicate the significance at P = 0.05 according to DMRT between mono-cultured and intercropped plants.

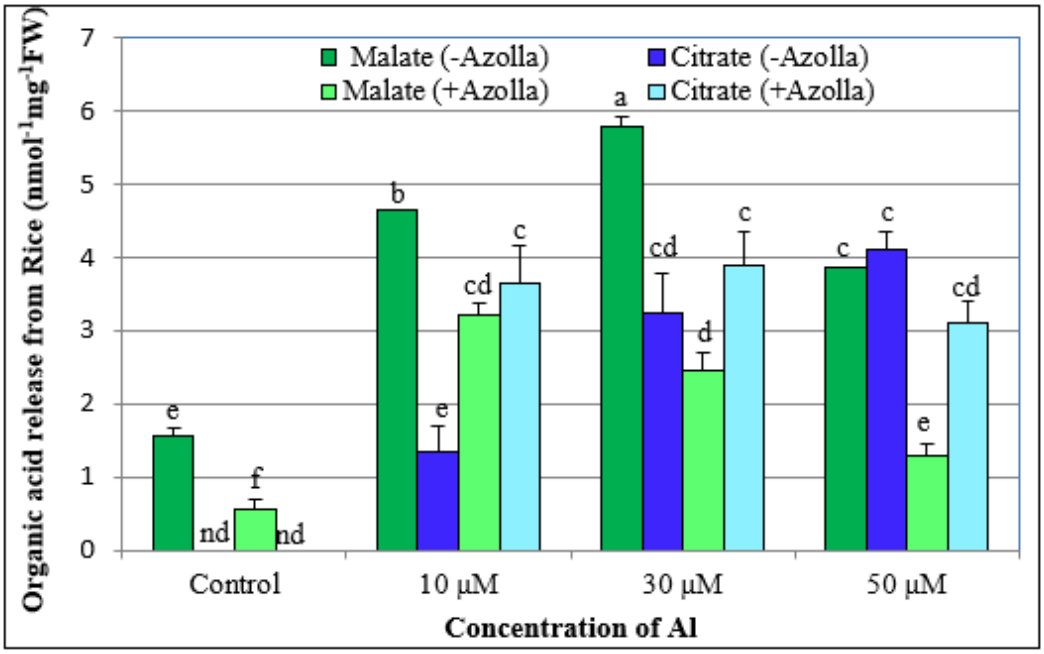


Fig.4. Variations in the organic acid release from rice with and without *Azolla* plants under different Al stress conditions. Each value indicates the mean ± SE (n = 3). The lower-case alphabets indicate the significance at P = 0.05 according to DMRT between mono-cultured and intercropped plants. *nd*-not determined.

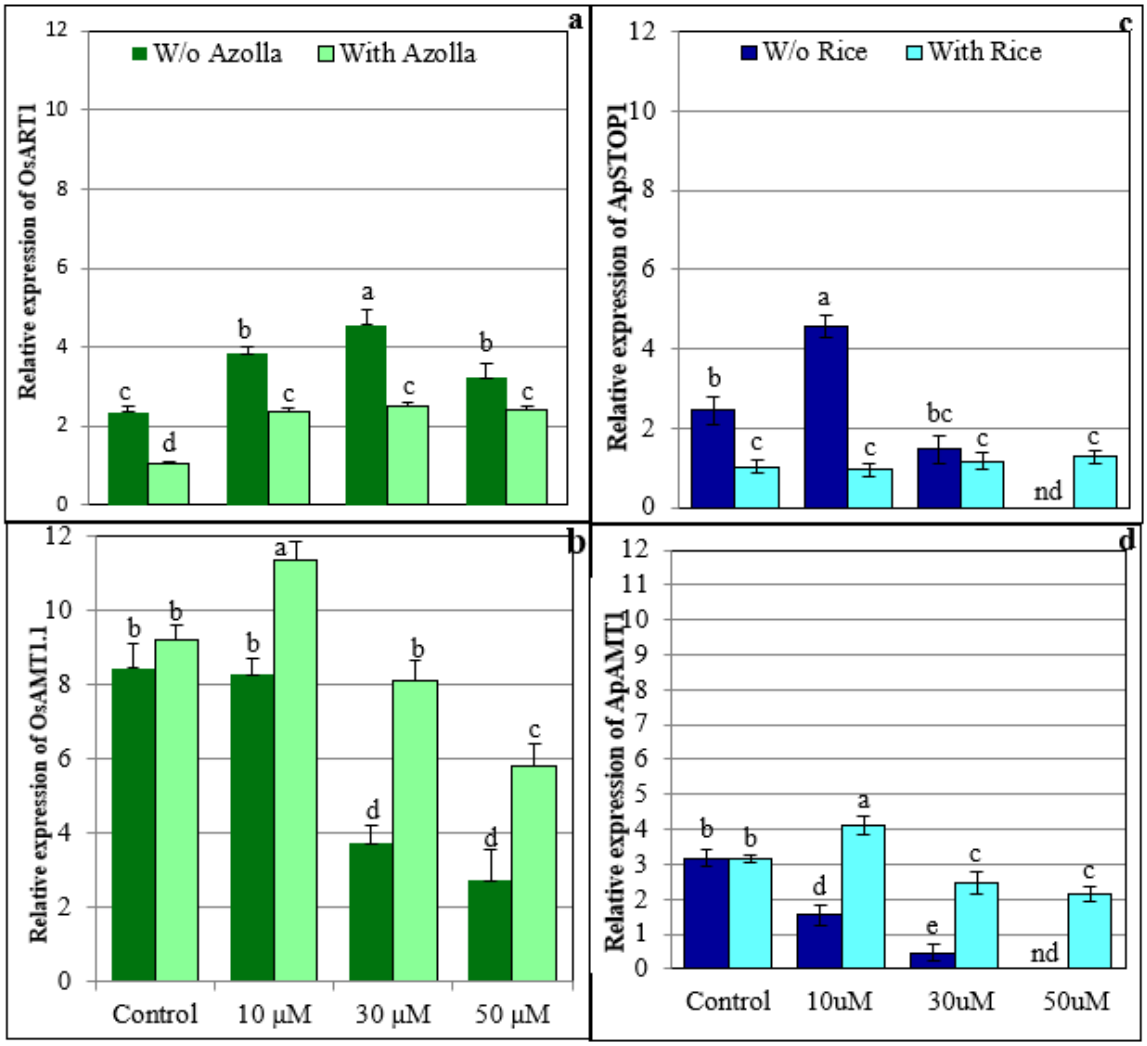


Fig.5. Variations in the Al stress induced expression profile of STOP1 and AMT1 genes of Rice (OsART1 [a] and OsAMT1.1 [b]) and *Azolla* plants (ApSTOP1 [c] and ApAMT1 [d]) under heterogenous and homogenous culture conditions. Each value indicates the mean ± SE (n= 3 - 5). For each gene, all the mean values from both the treatments were used for the DMRT based significance analysis. The lower-case alphabets indicate the significance at P = 0.05 according to DMRT between mono-cultured and intercropped plants. *nd*-not determined.

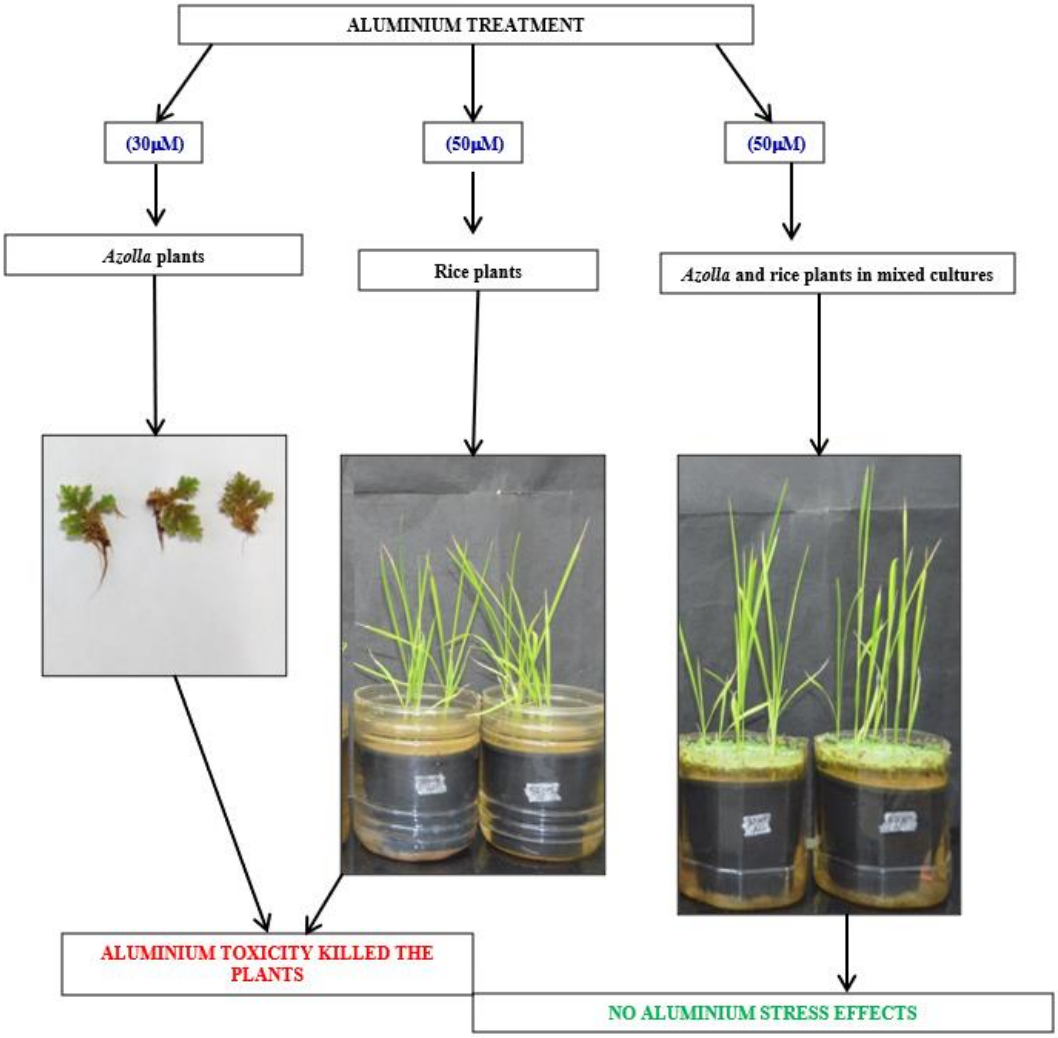


Fig.6. The advantages of mixed culture growth of *Azolla* and rice plants with special reference to Al stress and acid soil stress condition.

Objective 2

4.2 Characterization of Ammonium transporter gene (*ApAMT1*) of *Azolla pinnata*

4.2.1 Molecular cloning and sequence details

The full-length *ApAMT1* cDNA was obtained by degenerate PCR and RACE (Rapid Amplification of cDNA Ends) methods (degenerate primer: Fw-ctctcctattacctcttcggcttcgctttc; Rv-cccaagcsgacacgaacccgcart).

The gene-specific primers for 3'RACE and 5'RACE were designed by following the sequence of degenerate PCR products (5GSP1: cacggccgggtgctcggtcgtcga [981]; 5GSP2: tgggtgggtcaccgcgaccatggc [1281]; 3GSP1: cacggggtacacgaaccccgtgagga [481] and 3GSP2: ccgaacaagtagtaggagagcccgc [251]). The amplified fragments were aligned and the final sequence of *ApAMT1* was obtained by using CLC sequence viewer (Qiagen, Germantown, MD, USA). Further, the amplified cDNA fragments were cloned in the pBI121 cloning vector (Takara) and sequenced (Primers: Fw-atgaataagcaacgaccaaaattcaacaa; Rv-ttaagtagcgcatccccacgtg). Our results showed that the coding sequence of cDNA of *ApAMT1* consists of 1506 bp encoding 501 amino acid residues (**Fig. 8a**). Phylogenetic analysis indicated that *ApAMT1* is more closely related to other pteridophytes (**Fig. 8b**).

4.2.2 *In Silico* Secondary structure prediction and domain analysis of ApAMT1 protein

We used DeepTMHMM and HMMTOP tools to assess the number and type of transmembrane helices found in ApAMT1.2. The result in **Fig. 8a** shows that ApAMT1.2 is a multi-spanning protein with 11 transmembrane alpha helices, containing both hydrophobic and hydrophilic amino acid side chains. The N-terminus is found outside of the membrane while the C-terminus is found in the cytoplasm.

The PSI-BLAST alignment plot of ApAMT1.2 shows 1 strong peak and 3 relatively weaker peaks that indicate their putative domain boundaries, but only 3 domain boundaries have been predicted at 87, 227, and 388. This indicates that the second peak between 100-150 residues is probably a very long linker protein. In order to confirm this, we used PHYRE2 protein fold recognition server and the resultant Fig. clearly shows an extra-long linker chain between 97-132 amino acid residues. The signal peptide is predicted to be 1-13 amino acid residues long, present at the N-terminus.

Another web-based application Protter (Omasits et al., 2013) gives us an insight into five N-glyco motifs present at 7, 39, 188, 278 (outside the membrane), and 482 (inside the membrane) residues respectively. N-glycosylation of proteins occur at asparagine residues in the consensus sequence N-X-S/T (N=amide nitrogen of Asparagine; X≠P; S/T=Serine/Threonine), where an oligosaccharide, also called glycan, is attached to a nitrogen atom. This post-translational modification is catalyzed in the ER to modulate the folding of substrate protein, further increasing the range of its substrate, stability and protein-protein interactions (Strasser, 2016; Breitling and Aebi, 2013). Reports in *Arabidopsis*, rice and many plant species suggest that N-glycans play important role in growth of the plants during various stress conditions (Strasser, 2021).

4.2.3 *In Silico* Ramachandran Plot Analysis

Ramachandran plot of ApAMT1.2 (using WHAT IF server) indicates that for, 95.683% (399), 3.118% (13) and 1.199% (5) residues belong to the highly preferred regions (shown as Green crosses), preferred regions (shown as Brown triangles) and questionable regions (shown as Red circles) respectively. The highly preferred observations are located in conformations with delta ≤ -2 in the 2^{nd} and 3^{rd} quadrant.

4.2.4 *In Silico* Protein-protein interaction

Among the most basic interactors of ApAMT1.2 are nitrate transporters NRT1.1, NRT2:1, NRT2.2, nitrite reductase NIR1, nitrate reductase NIA1, glutamate synthase GLU1, glutamine sensing regulator GSR2, globin GLB1, glutamine synthetase GLN1;5 and AT2G28990 (Leucine-rich repeat protein kinase family). All these interactions are involved in nitrogen synthesis and transport in various forms in plants, thereby giving strong evidence that our protein is involved in ammonium transport. It also interacts with several transport proteins such as high-affinity nitrate transporter WR3, nitrate reductase NIA2, NADH-GOGAT GLT1 (highly expressed in roots), glutamine synthetase GS2, glutamate dehydrogenase GDH1, GDH2 as well as GDH3; glutamate:glyoxalate aminotransferase GGAT1 (required during photorespiration), and N-acetylglutamate kinase (NAGK) (modulates nitrogen use in plants). Thus, AMT1.2 in *Azolla pinnata* is also linked with various proteins responsible for nitrogen metabolism in plants.

4.2.5 *In Silico* Protein subcellular localization

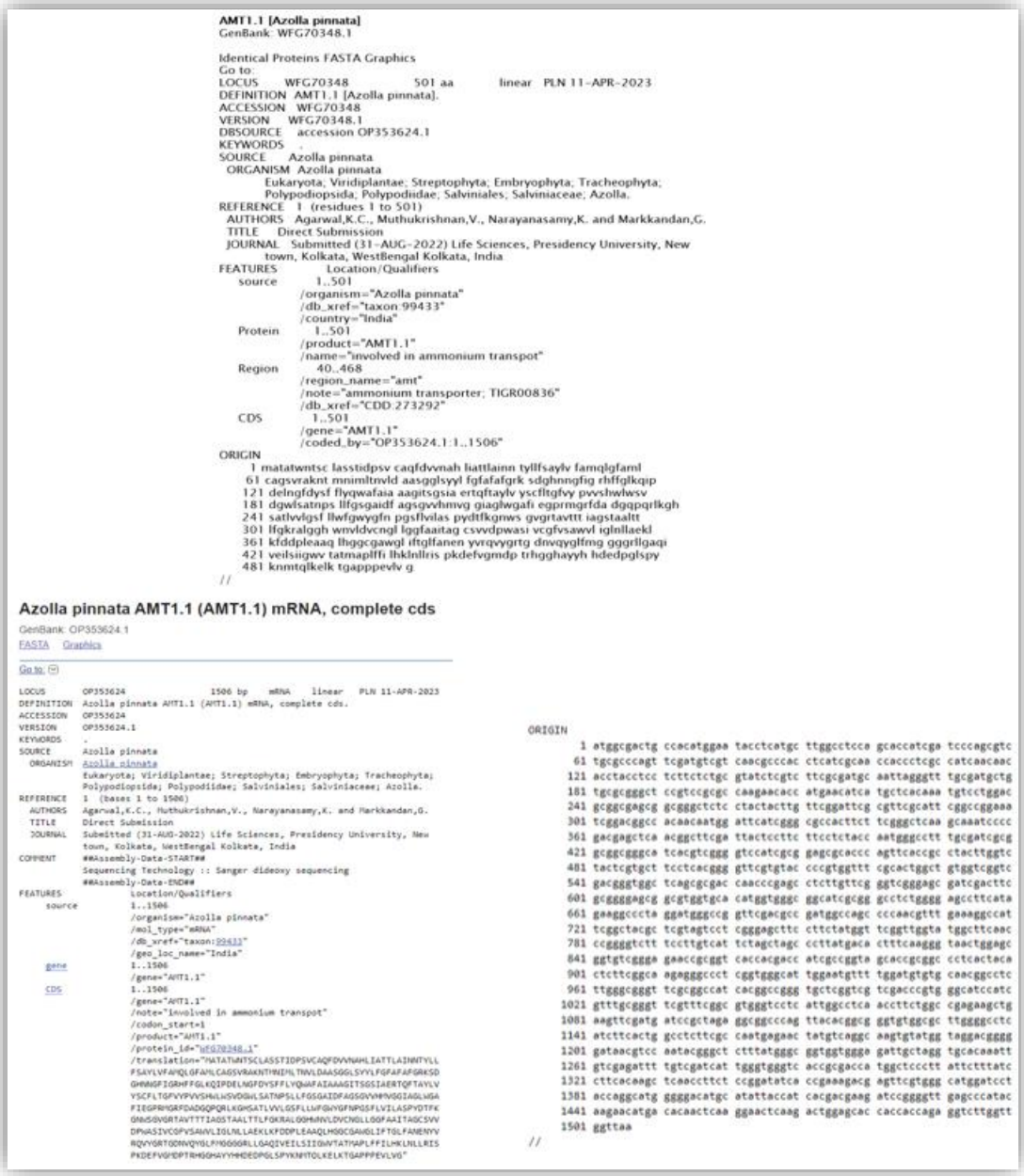
The site of protein is localized in chloroplast. This states that ApAMT1.2 functions mostly in the leaves of *Azolla pinnata* as a source of nitrogen for chlorophyll biosynthesis during photosynthesis.

4.2.6 Phylogenetic Analysis

AMT1 of *Azolla pinnata* shares the most recent common ancestor with other pteridophytes like *Adiantum nelumboides*, *Ceratopteris richardii* and *Selaginella moellendorffii* as well as with a bryophyte *Ceratodon purpureus*. ApAMT1 becomes the outgroup member as it diverges early and evolves, probably in course of Azolla becoming a complete aquatic plant. Other plants in the common group are terrestrial with only *Ceratopteris richardii* being semi-aquatic to aquatic in nature. In dicots, AMT1 of *Nicotiana tabacum* also shows sequence similarity with ApAMT1 (**Fig. 8b**).

4.2.7 *In Silico* Homology Modelling

The sequence of ApAMT1 matches confidently (indicated by red lines) with the entire chain of 11 different proteins with fold library entries c6eu6A_, c2b2hA_, c5aezA_, c5aexB_, c2nuuF_, c5aexJ_, c3c1iA_, c3b9yA_, c3hd6A_, c8csxQ_ and c7v0sK_ (where c indicates chain of protein from PDB; 6eu6 indicates the PDB identifier; and A indicates chain identifier). One domain d1u7ga_ from PDB identifier 1u7g chain A also matches confidently with our transporter protein. All these matching sequences are ammonium transporter proteins found in the membrane of fungi, bacteria, yeast as well as in blood group of humans. These are evident results with a 100 % confidence similarity that our protein ApAMT1 is a membrane bound ammonium transporter protein belonging to the Rhesus superfamily (Ludewig et al., 2007).



AMT1.1 [Azolla pinnata]
GenBank: WFG70348.1

Identical Proteins FASTA Graphics
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REFERENCE 1 (residues 1 to 501)
AUTHORS Agarwal,K.C., Muthukrishnan,V., Narayanasamy,K. and Markkandan,G.
TITLE Direct Submission
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Azolla pinnata AMT1.1 (AMT1.1) mRNA, complete cds

GenBank: OP353624.1
FASTA Graphics

Go to:

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VERSION OP353624.1
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REFERENCE 1 (bases 1 to 1506)
AUTHORS Agarwal,K.C., Muthukrishnan,V., Narayanasamy,K. and Markkandan,G.
TITLE Direct Submission
JOURNAL Submitted (31-AUG-2022) Life Sciences, Presidency University, New town, Kolkata, WestBengal Kolkata, India
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Fig.7 *ApAMT1* DNA and protein sequence.

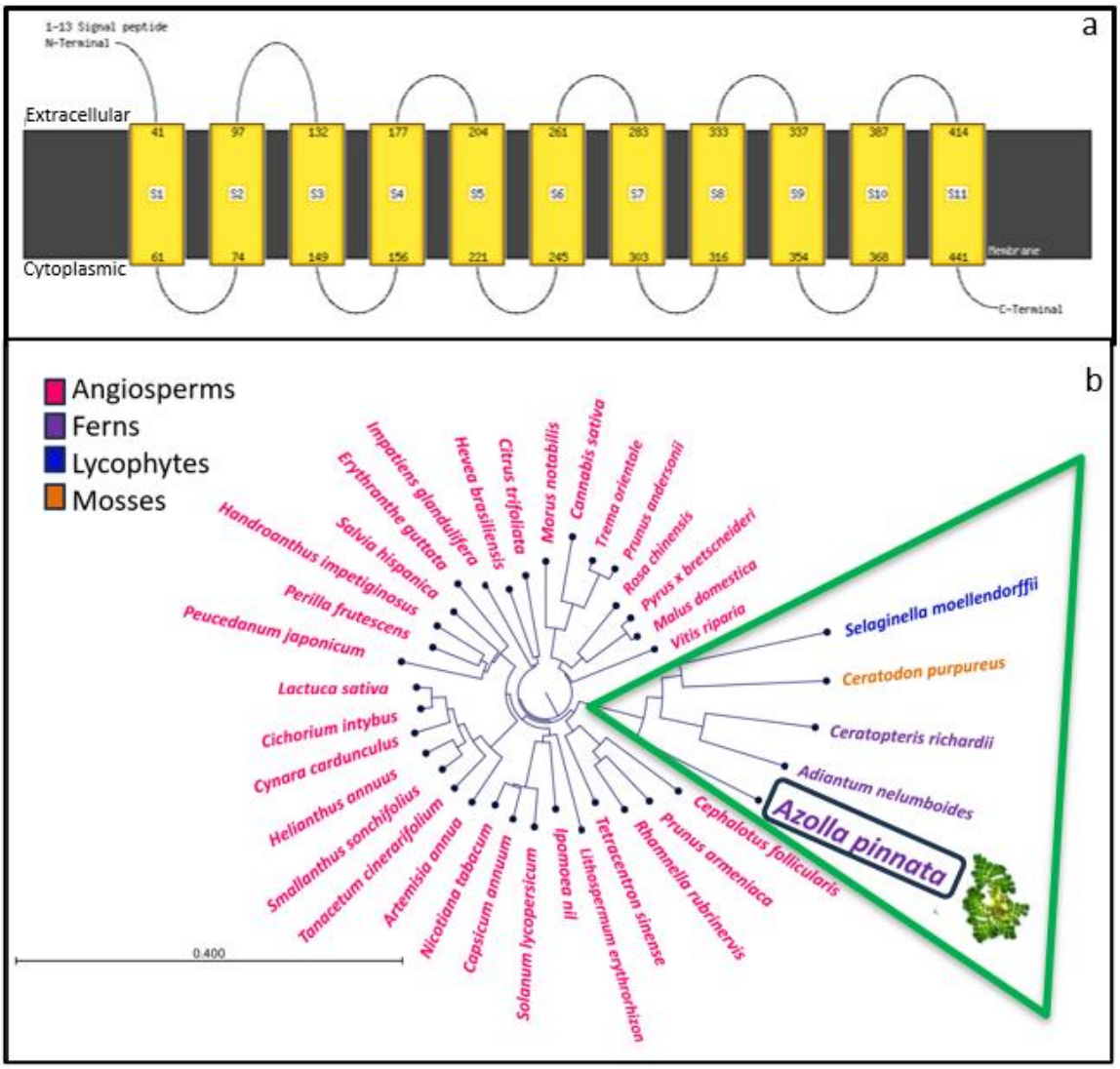

Fig.8 (a) ***Transmembrane helix prediction*** **using Phyre2 web based tool for** ***ApAMT1*** **sequence.** ***ApAMT1*** **sequence and the set of homologues detected by PSI-Blast are processed by a Support Vector Machine (a powerful machine learning tool) to determine the transmembrane helices and predict their topology in the membrane. For this Phyre2 uses memsat-svm. The extracellular and cytoplasmic sides of the membrane are labelled and the beginning and end of each transmembrane helix (illustrated with a number) indicates the residue index. (b)** ***Phylogenetic tree analysis*** **of** ***ApAMT1*** **with different plant species. The maximum-likelihood method was used to construct the tree.**

Objective 3

4.3 Overexpression of *ApAMT1* in Rice

We successfully cloned the *AMT1* from *Azolla pinnata* plants and overexpressed it in IR-64 rice plants using pBI121 plasmid vector using *Agrobacterium* transformation method (**Table 2**). Seeds were harvested from transgenic plants and PCR positive seeds were selected (**Fig. 9**) for studying the variation in the expression of *AMT1* under different stress treatments.

Table 2. Transformation efficiency rate achieved after *Agrobacterium* mediated IR-64 seed transformation procedure

Total number of healthy IR-64 rice seeds inoculated	1650	100%
Total number of seeds that formed callus/ Number of calli infected with Agrobacterium sp.	1287	78%
Total number of plant growth in selection media	36	2.8%
Total number of plants that are PCR-positive	9	25%
Total number of plants that produced seeds	4	44.44%
Total number of Transgenic seeds (T1) obtained	160	
TRANSFORMATION EFFICIENCY	**0.699%**	

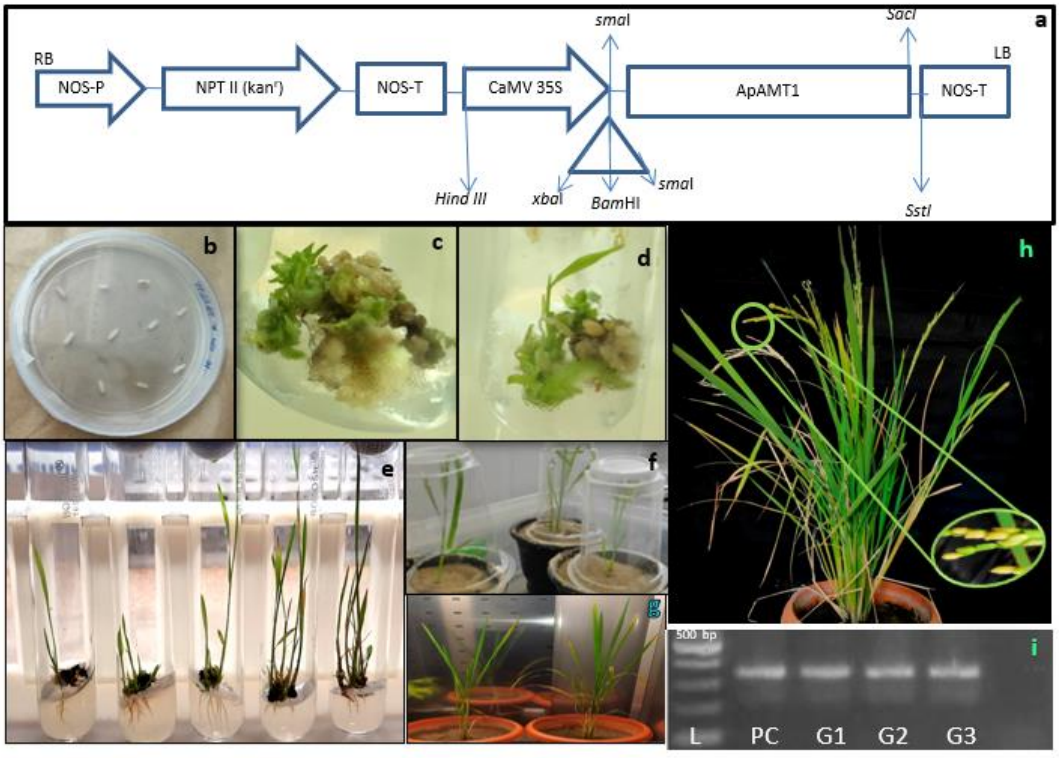

Fig.9 ***Details of the overexpression vector and genetic transformation methodology used.*** **a)** ***ApAMT1*** **gene was placed under the control of CaMV35S promoter for its overexpression in pBI121 vector with** ***nptII*** **selection marker. b) Seed explants were used for callus induction. c) Callus induction was achieved by using MS media fortified with 2,4-D & BAP. d) Shoot induction was achieved by using MS media fortified with NAA, BAP, Kinetin & GA_3. e) Root induction was achieved by using MS media fortified with IBA. f) and g) After 30 days of root formation, acclimatization was done by using transparent plastic containers under greenhouse conditions. h) Plants were adapted to natural environment and seeds were germinating. i) Harvested seeds were further used for PCR confirmation.**

4.4 Characterization of *ApAMT1* in Control and Transgenic Rice Plants

To analyse the impact of *ApAMT1* transgene in rice plants, we challenged the T1 seedlings with different concentrations of Ammonium nitrate (AN: 0.4, 4.0 and 20.0 mM). The nitrogen use efficiency (NUE) of these transgenic plants was measured by growing the seedlings for 10 to 14 days by using glass containers fortified with sterile soil. Firstly, we measured the variations in the morphological parameters of AMT1 to analyze the NUE. Overall, the transgenic plants maintained under low and moderate amount of AN, displayed significant growth variations when compared with high amount of AN treatment. The transgenic plants displayed significant variations in the shoot height under three different concentrations of AN (**Fig. 10**). Importantly, under normal conditions (AN: 4.0 mM), shoot height is lower than that of transgenic plants maintained under 0.4 mM or 20 mM of AN treatment.

Furthermore, we studied the variations in the root traits like total root length, root hair length and root hair density in the AMT1 transgenics. The total root length of AMT1 transgenics displayed very long root phenotypes when compared with WT plants at 4.0 mM AN treatment. Similar results were recorded in low and high AN treatment. Interestingly, the density of root hairs was higher in transgenic plants under low AN treatment (0.4 mM treatment) when compared with WT. Further, 4.0 and 20.0 mM AN treatment significantly increased the root hair density in the WT when compared with transgenics. The above results indicated that AMT1 expression enhances the root hair density under low AN treatments. Moreover, transgenic plants displayed longer root hairs when compared with WT plants at 4.0 and 20.0 mM AN treatment. Besides, high AN treated WT and the transgenic plants displayed comparable phenotypes. These results indicate that root hair length is negatively regulated by high AN content. In continuation of these results, we further checked the expression profile of AMT1s and biochemical stress markers. In transgenic lines, ApAMT1 expression increased significantly under increasing concentrations of AN when compared with the expression of OsAMT1 of WT plants (**Fig. 11**). Interestingly, the expression levels of OsAMT1 in transgenic and WT plants are comparable. The amount of biochemical stress markers like H_2O_2 and proline in transgenic plants was high at high AN concentration. These results specify that increased supplementation of AN causes stress to both WT and transgenic plants due to ammonium toxicity. On analyzing the variations in the photosynthetic values by means of Chl a fluorescence parameters, we found that Fv/Fm ratio of transgenic or WT plants at low and moderate levels of AN are not affected (**Fig. 12**). Surprisingly, high AN (20.0 mM)

treatment displayed decreased values that signifies a disturbance in the photosynthetic apparatus. Further, OJIP curve of dark-adapted leaves displayed that transgenic plants maintained under 0.4- and 4.0-mM AN displayed significantly higher values than that of WT plants.

Taken together, the *ApAMT1* transgenic rice plants maintained under low and moderate level of AN displayed several agronomically important traits which are important for NUE. Hence, we believe that the overexpression of *ApAMT1* in rice or other crop plants will be definitely useful to improve the plant growth or yield under low or moderate AN concentration.

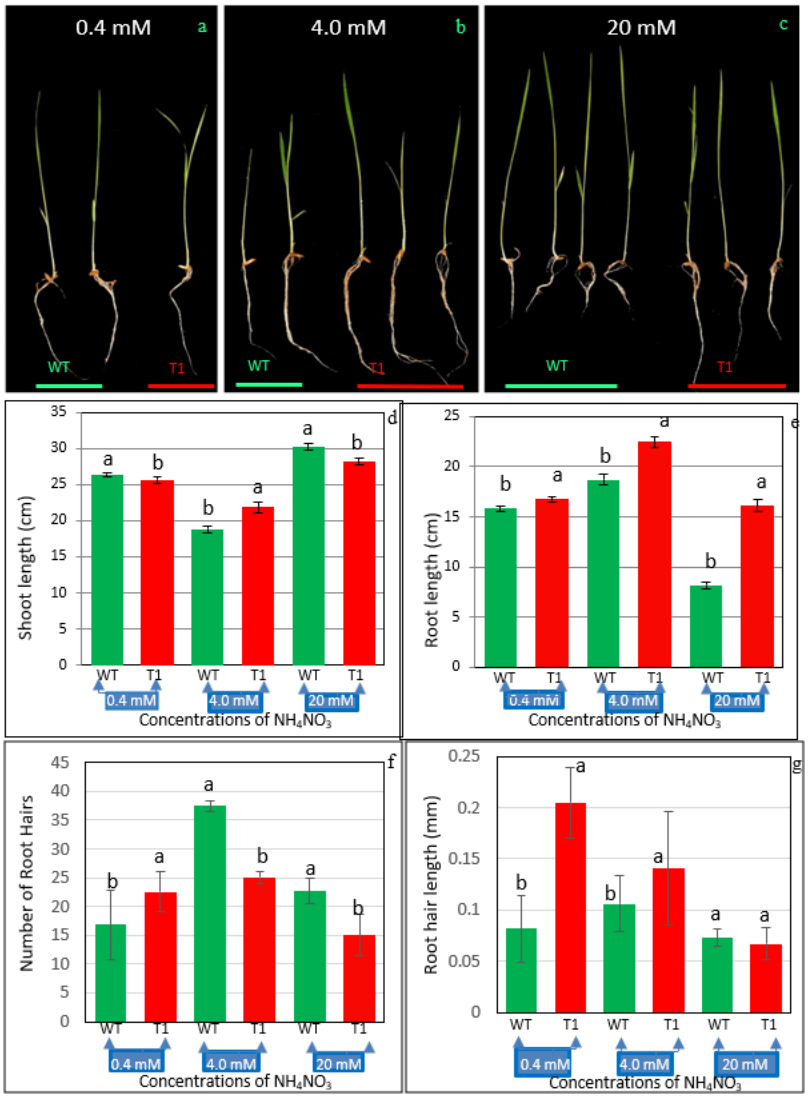

Fig.10 a-c) Comparative analysis between wild type (WT) and transgenic (T1) IR64 plants at pH 5.75 supplemented with different ammonium concentrations d) Variations in shoot length (in cm) e) Variations in root length (in cm) f) Variation in Number of Root Hairs per mm length after 8 weeks of experimental set-up. g) Variation in Length of Root Hairs (in mm) after 8 weeks of experimental set-up. Each value indicates the mean ± SE (n=3). Mean values followed by the same alphabets are not significant at P = 0.05 according to DMRT.

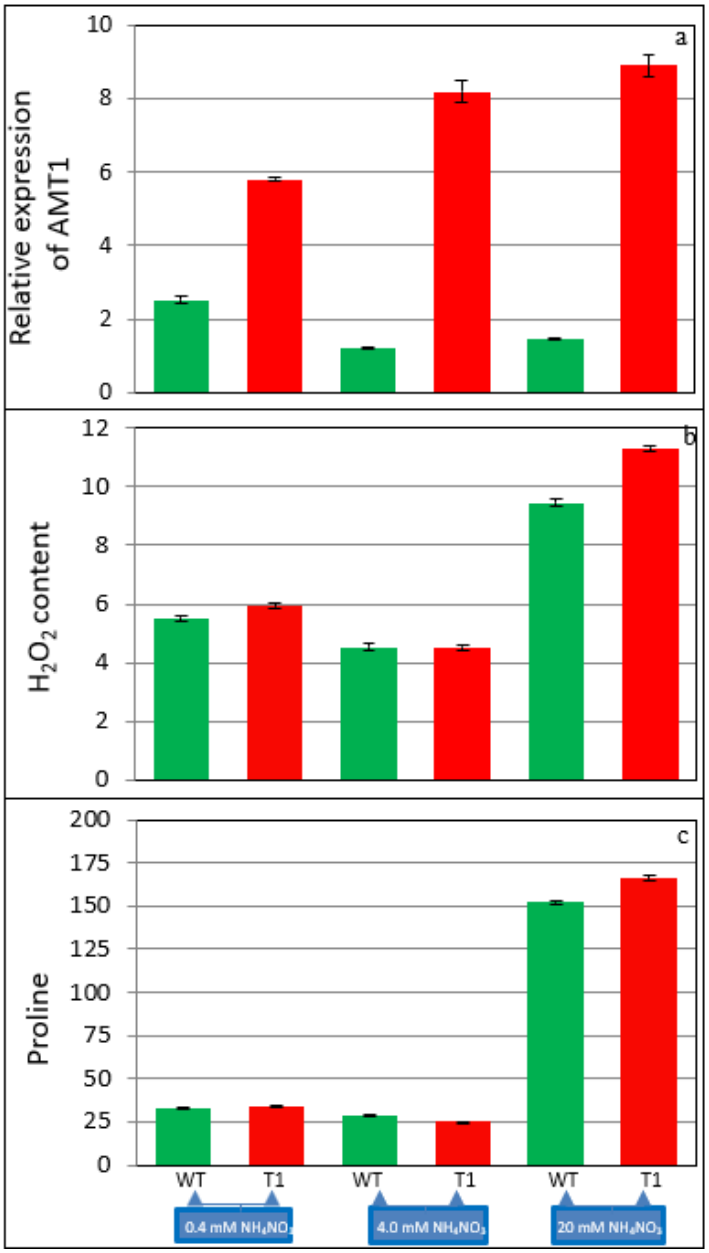

Fig.11 a) ***Expression profile analysis of AMT1 transgenics*** **under different nitrogen supplementation conditions b)** ***Variation in*** H_2O_2 **c)** ***Variation in Proline concentration*** **(nmol/mg FW) after 8 weeks of experimental set-up. Each value indicates the mean ± SE (n=3). Mean values within a treatment followed by the same alphabets are not significant at P = 0.05 according to DMRT.**

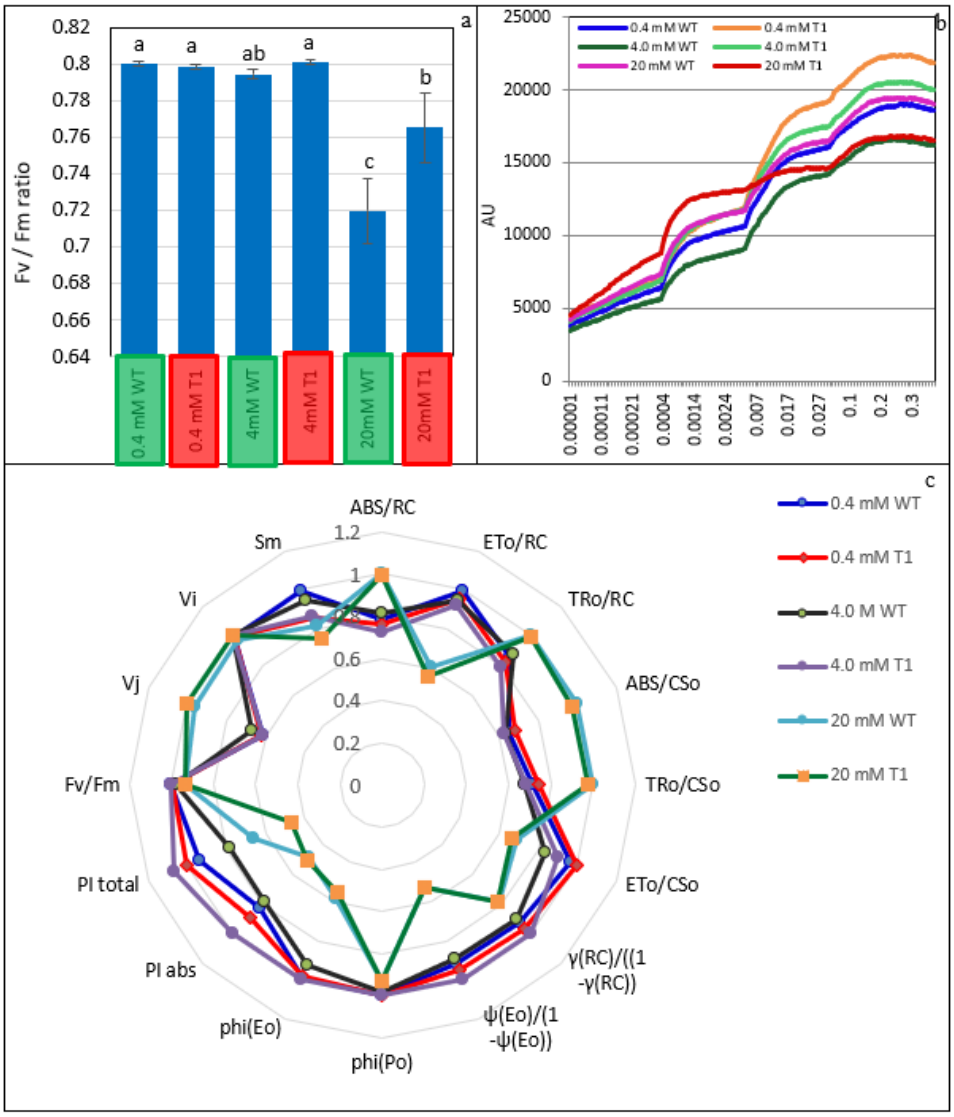

Fig.12 ***Variations in the Fv/Fm ratio, OJIP curve and Chl a fluorescence parameters in transgenic rice plants overexpressed with ApAMT1 gene*****. a) ApAMT1 over-expression Fv/Fm ratio. b) Chlorophyll-a fluorescence kinetics of the OJIP curve of dark-adapted leaves of control and transgenic IR-64 rice plants. Transient curve of each line represents the average of 10 measurements per treatment. c) Radar plot showing various technical fluorescence parameters. Each line represents the average of 10 measurements per treatment.*** ***[ABS /RC = total absorption of antenna chlorophylls per active RC; ET0/ RC = electron transport flux (further than QA) per active RC ; TR0/RC = trapped energy flux (leading to QA reduction) per RC ; φPo =maximum quantum yield for primary photochemistry ;φEo =quantum yield for electron transport (ET) ; γRC/(1-γRC) = QA-reducing RCs per PSII antenna Chl (reciprocal of ABS/RC) ;ψ0/(1 − ψ0) = efficiency with which a trapped exciton transfers an electron to the photosynthetic ET chain further than QA −;ABS/CSo = absorption flux per excited cross-section ;TRo/CSo = trapped energy flux per excited cross-section ;ETo/CSo = electron transport flux per excited cross-section ;PI abs= performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors ;PI total =performance index (potential) for energy conservation from exciton to the reduction of PSI end acceptors; Fv/Fm=Maximum potential quantum efficiency of PSII; Vj: variable fluorescence; Vi: initial fluorescence; Sm: energy necessary for closure of all reaction centres (RCs)]***

5.0 CONCLUSION

Incorporating *Azolla* plants in rice cultures reduced Al stress effects in both plants. Biochemical studies also demonstrated that heterogeneous cultures of *Azolla* and rice plants are not remarkably affected under low and moderate Al stress and that Al entry was inhibited. In addition, expression level of rice *ART1* gene was considerably higher than *Azolla STOP1* gene. ApAMT1 is a chloroplast-bound protein, involved mainly with ammonium transport, nitrogen metabolism, and other photosynthetic activities in plants. It belongs to the AMT1,2 family of ammonium transporters, which are found on root endodermis or cortex and have the highest ammonium uptake rate among other AMT1 family members. They also help to combat stress as they are involved in all the important metabolic functions in *Azolla pinnata.* AMT1 can help reduce the use of nitrogen fertilizers by increasing the NUE of crop plants, further increasing crop productivity. This suggests that *Azolla* plants can be rationally utilized in rice fields under acidic soil conditions to achieve Al tolerance and reduce Al accumulation in Al-sensitive rice varieties. AMTs are activated when external concentration of ammonium is low, as evident from our results. At higher concentration of ammonium, transgenic rice plants utilize ammonium by enhancing GABA shunt pathway thereby reducing ammonium toxicity and developing longer roots to combat the stress. Thus, *AMT1* from *Azolla pinnata* can be used for genetic engineering of crop plants to utilize ammonium even at higher concentrations in acidic soil and hence, reduce its toxic effects.

6.0 SIGNIFICANCE OF THE STUDY

- It highlights the importance of growing *Azolla pinnata* in rice fields for decreasing Aluminium toxicity caused due to acid soil.
- This is the first study on effects of Aluminium stress on growth and development of *Azolla pinnata* plants.
- It the first initiative to analyse the effects of Aluminium on *Azolla* and rice plants under intercropped conditions.
- The expression patterns of *AMT1* and *STOP1* genes indicated that both the plant species were not affected by Al under intercropped conditions.
- *Azolla pinnata* fails to grow over 30µM Al in monoculture. In intercropped cultures, both the plants exhibited reduced stress phenotypes.
- *ApAMT1* belongs to the AMT1,2 family of ammonium transporters.
- cDNA sequences of *ApAMT1* consists of 1506 bp encoding 501 amino acid residues.

- Phylogenetic analysis of *ApAMT1* indicated that it is an outgroup member, closely related to other pteridophytes, as it diverges early and evolves, probably in course of *Azolla* becoming a complete aquatic plant.
- *ApAMT1* is a chloroplast-bound protein, involved mainly with ammonium transport, nitrogen metabolism, and other photosynthetic activities in plants.
- *ApAMT1* can be used for genetic engineering of crop plants grown in acidic soil and hence, reduce the toxic effects of ammonium in plant cells.

7.0 FUTURE ASPECTS

Analysis of yield parameters of *ApAMT1* transgenics under field condition.

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ABBREVIATIONS

Al^{3+} : Aluminium

$AlCl_3$: Aluminium chloride

ALMT1 : Aluminium activated Malate Transporter 1

AMT1: Ammonium transporter

AMT/MEP/Rh : Ammonium Transporter/ Methylamine Permease/ Rhesus protein superfamily

ApAMT1 : AMT1 gene from *Azolla pinnata*

Ap : *Azolla pinnata*

ART1 : Al resistance transcription factor 1

β-actin : Beta actin

CBL : Calcineurin B-like

cDNA : complementary Deoxy ribonucleic acid

Chl : Chlorophyll

CIPK23 : CBL-Interacting Protein Kinase 23

DMRT : Duncan's multiple range test

dT : deoxy Thymidine

EDTA : Ethylenediamine tetraacetic acid

H^+ : protons

h/day : Hour per day

$h^{-1}g^{-1}$: Per hectare per gram

H_2O_2 : Hydrogen peroxide

IR : International rice

M : Molar

MATE : Multidrug and Toxic compound Extrusion

MDA : Malondialdehyde

mg : Milligrams

min : Minutes

ml / mL : Millilitres

mM : Millimolar

mol/g : Mole per gram

N / N_2 : Nitrogen

$Nh^{-1}g^{-1}$: Nitrogen per hectare per gram

nm : Nanometres

NUE : Nitrogen use efficiency

Os : *Oryza sativa*

P : Probability

pH : Potential of hydrogen

PVPP : Polyvinyl polypyrrolidone

RNA : Ribonucleic acid

ROS : Reactive oxygen species

rpm : Rotations per minute

SPSS : Statistical package for the Social Sciences

STOP1 : Sensitive To Proton rhizotoxicity1

TBA : 2-Thiobarbituric acid

TCA : Trichloroacetic acid

µmol $m^{-2}s^{-1}$: Micromoles per metre square per second

µM : Micromolar

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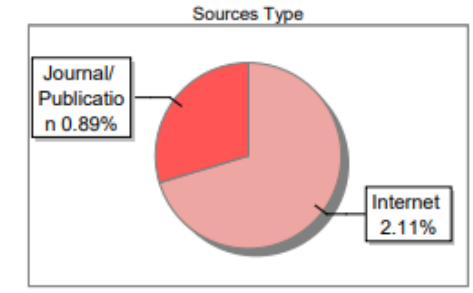

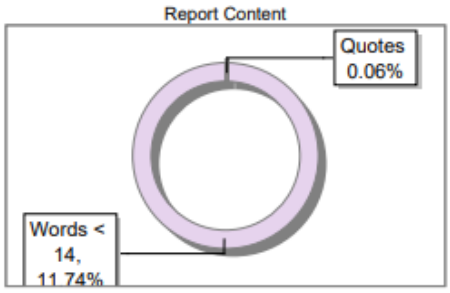

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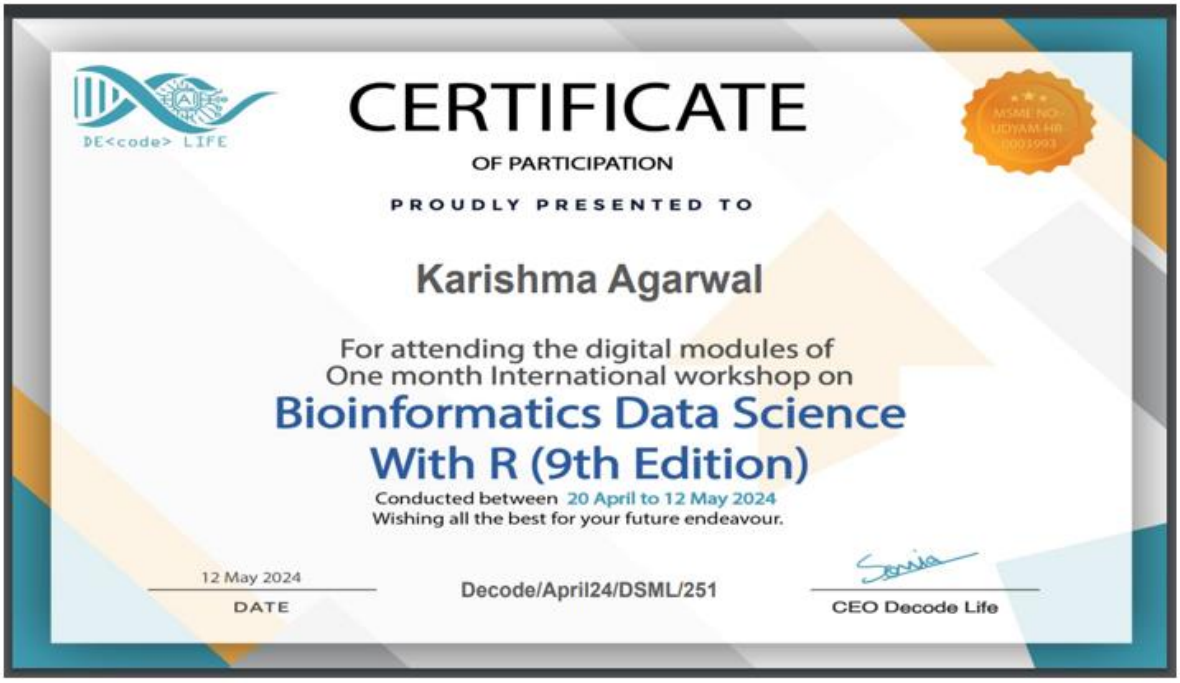

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Azolla pinnata redefines its importance in rice fields as it alleviates aluminum toxicity and low pH stress

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ABSTRACT

Monoculture of rice seedlings or *Azolla pinnata* was challenged with different aluminum stress conditions, and both species showed significantly reduced total biomass, chlorophyll, root, and leaf length. Mixed cultures showed no stress phenotypes and notably enhanced growth parameters under low and moderate aluminum stress (10 and 30 µM). Discretely, *Azolla* plants failed to survive when grown at >30 µM aluminum treatment (pH 4.75) but sustained well when grown with rice plants. Importantly, both species accumulated less aluminum and more root exudates in mixed cultures of *Azolla* and rice plants. Furthermore, expression of *Sensitive To Proton rhizotoxicity1* (ApSTOP1 and OsART1) in both species declined significantly in mixed cultures than in monocultures. *Ammonium transporter 1* (ApAMT1 and OsAMT1.1) expressed significantly more in heterogeneous cultures, indicating that ammonium transport is unaffected. Our observations conclude that aluminum accumulation and stress effects significantly decreased in heterogeneous cultures when compared with homogenous cultures.

1. INTRODUCTION

Acidic soil is one of the most common issues faced by farmers in agricultural fields, where major crops are grown [1]. Acidic soil environments are stressful as well as toxic to plants [2]. When soil and surface water are acidified to a pH below 5.0, monomeric aluminum hydroxides and Al-containing minerals are dissolved to form polynuclear aluminum species and trivalent aluminum (Al^{3+}) that are readily absorbed by plants [3,4]. However, soluble Al like aquo/hydroxo-Al complexes have deleterious effects on plant growth and survival, primarily by hindering the uptake of nutrients from the soil [5,6]. In plant tissues, Al content ranges from 0.2 to 1000 mg/kg, and variations in Al accumulation are regulated by different factors such as plant species, soil type, and soil nutrients. It has been reported that excess Al is found in those plants that grow in acidic soils mainly [7]. Al exists in soil in the form of various ions, among which Al^{3+} is the most toxic [6,8]. Al also forms insoluble oxides and hydroxides at pH 5.0 to 6.2, as well as polynuclear dissolved species below pH 5.0, that determine Al toxicity [5,9].

In Arabidopsis, Al^{3+} ions are recognized and regulated by several genes, transcription factors, and transporters [10]. The principal functional tool for plant tolerance to Al^{3+} includes Al^{3+}-induced stimulation of transporters that accelerates the discharge of organic acids from the roots. When the amount of Al^{3+} increases in the soil, it is either chelated by organic acids that are excreted from the roots into the rhizosphere or Al enters the plant cells through the roots via absorption [11]. *Multidrug and Toxic compound Extrusion* [MATE] and *aluminum activated Malate Transporters* [ALMT1] are key transporters involved in the release of citrate and malate, respectively, in most plants. These transporters are regulated by the STOP1 system [12-14]. Rice plants displayed a high level of Al-tolerance when compared with other crop plants [15]. In particular, *sensitive to aluminum rhizotoxicity 1* [STAR1] and *sensitive to aluminum rhizotoxicity 2* [STAR2], which encode domains of a *bacterial-type ATP-binding cassette* [ABC] transporter, and *Al-resistance transcription factor 1* [ART1], a homologue of STOP1, are key genes involved in Al tolerance. These genes are mainly expressed in roots and regulate multiple genes for Al tolerance in rice plants [16]. The *Azolla* plant is a heterosporous aquatic fern characterized by high rates of multiplication and biological nitrogen fixation potential due to the presence of cyanobacterial symbionts. The cyanobacterial symbiont in *Azolla* can fix a significant amount of nitrogen [0.15–0.17 mg $Nh^{-1}g^{-1}$ of dry biomass] compared with *Rhizobia*, which fixes only 0.08 mg $Nh^{-1}g^{-1}$ of dry biomass in *Glycine max* [17]. In addition, studies have found that under stress conditions, *Azolla* can also increase the nitrogen use efficiency (NUE) of rice plants, thereby increasing grain yield [18].

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